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Isolation of Sperm Storage Tubules from the Uterovaginal Junction Mucosa of the Turkey

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ABSTRACT This study was performed to determine whether intact sperm storage tubules (SST) could be successfully isolated from the uterovaginal junction (UVJ) mucosa of the turkey. Large White BUTA hens were inseminated and euthanatized 24 to 48 h later. Oviducts were excised, UVJ tissue removed, and SST were procured by enzymatic digestion. Recovered SST were intact and contained motile sperm. The sperm were oriented with their acrosomes pointed towards the distal end of the SST, and their long axes in parallel with the long axis of the tubule's lumen. This method for the isolation of intact SST can be readily applied for *in vitro* culture studies as well as for the extraction of DNA and RNA from the SST epithelium.

(*Key words*: turkey, sperm storage tubules, sperm, uterovaginal junction)

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INTRODUCTION

Sperm storage tubules (SST) are structures found in the distal half of the oviduct of all avian species studied to date. These structures contain and store sperm, which are slowly released over time while the hen is in egg production to insure an adequate population of sperm at the site of fertilization (Bakst, 1993). Although detailed studies using histochemistry (Gilbert *et al*., 1968; Pal, 1977; Bakst, 1987) and electron microscopy (Van Krey *et al*., 1967; Tingari and Lake, 1973; Bradley, 1982; Schuppin *et al*., 1984) have evaluated the number, function, and histological characteristics of the SST epithelium and surrounding mucosa, very little is known about the physiological mechanisms of sperm storage in birds (see Bakst *et al*., 1994 for review).

Schuppin *et al*. (1984) speculated that the primary function of the SST epithelium was absorption because his transmission electron microscopy observations revealed limited ribosomal synthesis of secretory proteins. The SST do not have an exocrine function, and lack active secretory epithelium (Bakst, 1987). Sperm in the SST lumen are not embedded in nor bound to the apical region of the SST epithelium, but instead are intimately associated with the plasmalemma of adjacent sperm (Tingari and Lake, 1973; Bakst *et al*., 1994). Lipid material has been observed in the SST epithelium of chicken, turkeys, and Japanese quail (Bakst, 1987, 1993), which is thought to include cholesterol, cholesterol ester, and phosphatidyl ethanolamine (Wall, 1975). This lipid material may aid in the maintenance of the sperm plasmalemma, and protect against oxidative damage.

Quantification of the number of sperm contained within intact SST is difficult, as the sperm are often tightly packed together. Whole SST are rarely in one plane of a histological section, and the fixation and tissue preparation procedures may alter sperm numbers. A hemacytometer was used to count total sperm released by moderate grinding of turkey SST (Brillard and Bakst, 1990), by collagenase digestion (Brillard, 1993), or by homogenization (McLean and Froman, 1996) of chicken SST. Filling of SST has also been evaluated *in vitro*, using oviductal tissue explants (McLean and Froman, 1996).

Radiolabels and fluorescent stains improve visualization of sperm. Sperm stained with the nuclear fluorescent dye bisbenzimide (Hoechst 33342) were found in the distal end of the SST aligned in a parallel, head-to-head array (Bakst *et al*., 1994). Apparent stratification of radiolabelled sperm within the SST (Van Krey *et al*., 1981) gave credence to the hypothesis that the last sperm inseminated are the first to be released from the SST (Burke and Ogasawara, 1969; Compton *et al*., 1978).

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Abbreviation Key: BPSE = Beltsville Poultry Semen Extender; BUTA = British United Turkeys of America; HBSS = Hanks Balanced Salt Solution; SST = sperm storage tubules; UVJ = uterovaginal junction.

However, some authors refute this concept, citing lack of stratification seen within the storage tubules of zebra finches (Birkhead *et al*., 1988, 1990).

Our experimental objective was to develop a technique by which viable, intact SST could be isolated for subsequent study. This research was conducted using turkeys because a significant feature of the reproductive physiology of the turkey hen is her ability to store sperm for up to 70 d (Christiansen and Bagley, 1989). Thus, the SST of turkeys may be uniquely suited for such extended sperm storage. However, with minor modification, the procedure outlined here could be used in other species.

MATERIALS AND METHODS

Animals

Large White BUTA[2] breeder turkeys were maintained in an environmentally controlled house on a 14 h:10 h light:dark photoperiod and housed individually in cages (hens) or in groups of 8 to 10 in pens (toms). Feed and water were provided for *ad libitum* consumption. Semen was collected by abdominal massage, and 1×10^8 sperm were used for artificial insemination. Hens were euthanatized 24 to 48 h after artificial insemination by cervical dislocation. Eight hens were used for this study.

Sperm Staining Procedure

Semen was diluted 1:1 in Beltsville Poultry Semen Extender (BPSE).[3] Hoechst 33342[4] (20 μL diluted in 1.32 mL BPSE) was added at a ratio of 1:1. The stained sperm were placed on a rotating shaker at 22 C for at least 2 h prior to use (Bakst, 1994).

SST Isolation Procedure

The oviduct was isolated and removed from the reproductive tract immediately after euthanasia. The connective tissue surrounding the vagina and uterus was carefully removed by lifting and cutting the connective tissue away from underlying muscularis mucosa. The vagina and uterus were excised longitudinally, spread out on a dissecting board, and rinsed with cold PBS,[5] pH 7.4. Beginning at the vaginal end, the vaginal mucosa was scraped with a scalpel, through the uterovaginal junction (UVJ) until the uterus was reached. The isolated mucosa was spread out in a Petri dish containing PBS and viewed by stereomicroscopy (5×). The UVJ mucosa contained the SST embedded in longitudinal folds. The vaginal mucosa was removed and discarded, leaving only the UVJ mucosa containing the SST (Brillard and Bakst, 1990). The isolated UVJ mucosa was weighed (nearest milligram) and placed in a large glass Petri dish. Type XI collagenase[5] [0.01 g / mL Hanks Balanced Salt Solution (HBSS),[5] pH 7.4] was added (0.5 μL / mg mucosa). The tissue was minced in the collagenase with a scalpel into very small fragments and transferred into a 5-mL test tube. The glass Petri dish was rinsed with an equal volume of HBSS as that of collagenase (typically 250 to 500 μL), and this was added to the test tube. The tube was capped and placed horizontally in a shaking 37 C water bath for 10 to 15 min.

After 5 min, a 50-μL aliquot was removed and examined by stereomicroscopy to monitor the release of SST from the loose connective tissue. When approximately 70% of the SST were detached from the mucosa, the test tube was placed on ice, and 2 mL cold HBSS was added to stop enzymatic activity. The preparation was transferred to a small Petri dish, with additional HBSS added, and isolated SST were removed by successive aspirations with a 1 to 10 μL pipet tip.

RESULTS AND DISCUSSION

The method described here results in the successful isolation of intact SST from turkey oviductal mucosa. The average percentage recovery was 10 μg SST protein / g mucosal tissue. The SST obtained were long and coiled (Figure 1A). Some SST contained ciliated epithelium at their oviductal end, which could be seen beating at room temperature. The height of the SST epithelial cells ranged from 18 to 22 μm and the outside diameters of the tubules were approximately 45 to 50 μm, consistent with published findings (Schuppin *et al*., 1984; Bakst *et al*., 1994). Lipid material was present, and there was no obvious separation of cells or gaps. Sperm remained in the SST lumina throughout the isolation procedure, and were most prevalent in the distal ends of the tubules. The sperm were arranged with heads aligned at the distal end of the tubule, and the tails beat in a slow synchronous motion at room temperature. After fixation, sperm became coiled and were no longer motile (Figures 1B, C, D), indicating possible osmotic disturbance of the interior fluid composition of the SST. As the sperm did not coil when fixed separately, perhaps enzymatic dissociation from the oviductal epithelium disrupted the ability of the SST epithelium to regulate osmotic properties of interior fluids.

This method can also be amended to allow for the gross collection of multiple SST by simply stopping the isolation procedure after the enzymatic digestion, and prior to the aspiration of individual SST. However, fragments of partially digested mucosa and oviductal epithelium will remain in solution, along with the SST. This modification may be of use for quantification or other purposes.

For the past decade, several techniques and methodologies have been developed to assess sperm storage efficiency in the oviduct of various poultry species, such

[2]British United Turkeys of America, Lewisburg, WV 24901.
[3]Tri Bio Laboratories, Inc., State College, PA 16803.
[4]Molecular Probes, Inc., Eugene, OR 97402.
[5]Sigma Chemical Co., St. Louis, MO 63178-9916.

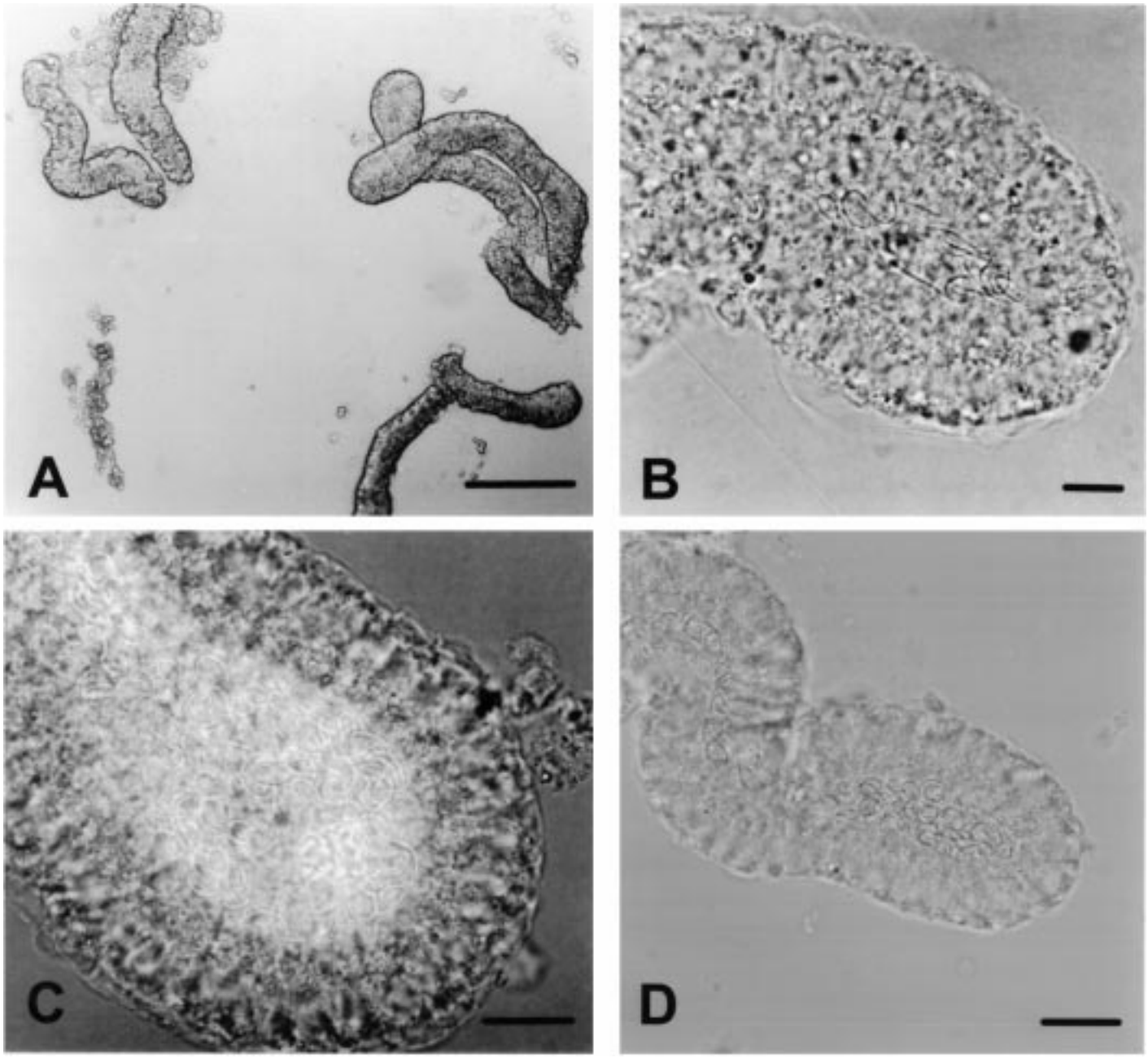

FIGURE 1. A) Isolated sperm storage tubules (SST), obtained by enzymatic dissociation from uterovaginal junction mucosa, viewed by light microscopy (20×) with a wet mount in Hanks Balanced Salt Solution (HBSS). Individual SST can be seen, along with fragments of epithelial cells. Cilia along the epithelial cell borders were beating. The lumina of the tubules, especially the distal ends, are filled with motile sperm, arranged with heads closely aligned. Bar = 100 μm. B and C) Isolated SST, viewed by differential interference contrast microscopy (100×). Preparation fixed in 4% paraformaldehyde, 20 m*M* EGTA 1:1 with HBSS for 24 h prior to photography. Fluorescent blue Hoechst-stained sperm can be seen filling the distal lumens of the tubules. Bars = 10 μm. D) Isolated SST, viewed by confocal microscopy with incidental light (92×). Preparation fixed in 4% paraformaldehyde, 20 m*M* EGTA 1:1 with HBSS, and mounted 1:1 with PBS, Dabco, and 40% glycerol. Sperm are visible in the distal lumens of the tubules. Bar = 20 μm.

as turkey (Brillard and Bakst, 1990), and chickens (Brillard, 1993; McDaniel *et al.*, 1997; Brillard *et al.*, 1998). However, none of these techniques are suitable for the study of sperm penetration or storage under *in vitro* conditions. The present work demonstrates that the recovery of significant populations of functional SST from a single hen is feasible; it therefore opens new possibilities for the study of mechanisms involved in the storage and release of spermatozoa under controlled environmental conditions in avian species.

The procedure outlined here is an important beginning to further studies of SST physiology. The SST isolated by the procedure described here have a normal morphology and maintain their content of sperm. This normality demonstrates the suitability of the SST for use in other applications, such as *in vitro* culture studies,

which would give a unique opportunity to evaluate sperm uptake and release from the SST. Because the SST are isolated in a relatively pure state, gross contamination from oviductal surface epithelial cells and mucosal cells can be avoided. Extraction of DNA and RNA from the SST epithelium can also be obtained, which may lead to the identification of genes and proteins involved in the subsistence of sperm in storage.

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